Two Nuclear Genes Confirm Mitochondrial Evidence of Cryptic Species within *Liriomyza huidobrensis* (Diptera: Agromyzidae)

SONJA J. SCHEFFER[1] AND MATTHEW L. LEWIS

Systematic Entomology Laboratory, PSI, Agriculture Research Service, USDA, Building 005, Room 137, BARC-W, 10300 Baltimore Avenue, Beltsville, MD 20705

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ABSTRACT Phylogenetic analysis using DNA sequence data from two nuclear genes was undertaken to investigate phylogeographic structure within the widespread leafmining pest *Liriomyza huidobrensis* (Blanchard). Parsimony analysis of 171 bp from β-tubulin (including an intron) and 921 bp from elongation factor-1α confirms previous findings from mitochondrial sequence data of deep phylogeographic structure indicative of cryptic species within *L. huidobrensis.* We resurrect the name *L. langei* Frick for the North American cryptic species and restrict the name *L. huidobrensis* to the South and Central American cryptic species. Results from nuclear genes also confirm previous results suggesting that recent invasions of this leafminer in many areas of the world are due to the spread of *L. huidobrensis* from South or Central America.

KEY WORDS *Liriomyza huidobrensis, Liriomyza langei*, pea leafminer, phylogeography, invasive species, elongation factor-1α

PHYLOGEOGRAPHIC ANALYSIS OF DNA sequence data has become a widespread and powerful tool for delineating and identifying species, particularly in groups where species may be morphologically cryptic (Avise 2000). Phylogeographic data have also been used to understand how geographic features influence divergence and to identify the geographic origins and mechanisms of spread of invasive species and disease organisms (Hall and Muralidharan 1989, Slade and Moritz 1998, Villablanca et al. 1998, Frohlich et al. 1999, Scheffer 2000). Sequence data from mitochondrial genes provides several advantages for phylogeographic studies and is commonly used (Avise 2000). However, several biological processes can cause results from only a single gene region to be misleading (Neigel and Avise 1986, Moore 1995, Doyle 1997, Hoelzer 1997). Corroboration of results from additional unlinked markers, such as nuclear genes, provides a firmer foundation for interpretations regarding species limits (Avise and Ball 1990).

Liriomyza huidobrensis is a polyphagous leafminer that feeds on a variety of plant species, including numerous vegetable and flower crops, in at least 14 families (Spencer 1990a, 1990b). The endemic range of *L. huidobrensis* appears to have been western North America, primarily California, and much of South America (Spencer 1973). It was not reported from Central America until 1983 (Costa Rica, Spencer 1983), but is now common on numerous crops in several Central American countries. During the past decade, *L. huidobrensis* has become globally invasive and can now be found in many greenhouses and vegetable and flower growing areas of Europe, Asia, Africa, and the Middle East (Spencer 1990b, de Goffau 1991, Bartlett 1993, Cheek et al. 1993, Weintraub and Horowitz 1995, Shepard et al. 1996, Scheffer et al. 2001).

Liriomyza huidobrensis was originally described from *Cineraria* in Argentina in the 1920s (as *Agromyza huidobrensis* Blanchard, 1926). It was present as a pest in California in the 1930s (Spencer 1973), although it was not recognized as *L. huidobrensis* at the time, and was formally described as *L. langei* Frick (1951) primarily on peas. The high degree of variation in the coloration exhibited by adult *L. huidobrensis* resulted in three additional junior synonyms being described in association with various crops (Spencer 1973). In Argentina, Blanchard described *L. cucumifoliae* Blanchard (1938) from melon and *L. decora* Blanchard (1954) from fava bean. In California, in addition to describing *L. langei*, Frick also described another junior synonym *L. dianthi* Frick (1958). Once the male genitalia of agromyzids were discovered to be important characters for species discrimination, the synonyms were discovered and sunk. Frick (1964) synonymized *L. dianthi* with *L. langei*, and Spencer (1973) synonymized *L. langei*, *L. cucumifoliae* and *L. decora* with *L. huidobrensis*, leaving *L. huidobrensis*, as currently defined, to represent one species ranging more or less throughout the Americas from California south to Argentina.

Despite its recognition as a single species, there has been some suggestion that the flies from California and the flies from South America may exhibit differences in host preferences, pest status, and insecticide resistance (Spencer 1973, Bartlett 1993, Weintraub

[1] E-mail: sscheffe@sel.barc.usda.gov.

Table 1. Source locality and host information for in-group specimens sequenced for β-tubulin

N		Country	Location[a]	Host plant[b]	Collector(s)
1	—	United States	Monterey County, CA	*Lactuca* sp. ("romaine")	Franklin Dlott, William Chaney
2	—	United States	Monterey County, CA	Lab Colony A	Franklin Dlott, William Chaney
2	—	United States	Monterey County, CA	Lab Colony B	Franklin Dlott, William Chaney
1	—	United States	Monterey County, CA	Lab Colony C	David Morgan, John Trumble
2	—	United States	San Diego County, CA	Lab Colony D	David Morgan, John Trumble
3		United States	Ventura County, CA	*Gypsophila*[b]	John Rogers
2		United States	San Luis Obispo County, CA	*Spinacia oleracea*	Earthbound Farms
2		United States	Monterey County, CA	*Lactuca* sp.	Franklin Dlott
3	—	United States	Hawaii, HI	*Apium* sp.	Robert Hollingsworth, Marshall Johnson
2	—	United States	Maui, HI	*Allium* sp.	Laura Minuto, Ronald Mau
2	—	Guatemala	Chimaltenango	*Pisum sativum*	Phillip Lamport, Steven Weller
2	—	Guatemala	Chimaltenango	*Vicia fava*	Phillip Lamport, Steven Weller
1	—	Guatemala	Chimaltenango	"weed in oak forest"	Phillip Lamport, Steven Weller
2	—	Ecuador	Carchi	*Solanum* sp.	Roger Williams
2		Colombia	unknown	*Chrysanthemum* sp.	APHIS PPQ
2		Colombia	unknown	*Aster* sp.	APHIS PPQ
1		Peru	Huanacayo	*Vicia fava*	Norma Mujica
1		Peru	Lima	*Lactuca* sp.	Norma Mujica
1		Peru	Carabayllo	*Allium cepa*	Norma Mujica
1		Argentina	Cordoba	*Beta vulgaris*	Graciela Valladares
1	—	Sri Lanka	Galpalama	*Allium* sp.	Gamini Herath, Anura Wijesekara
1	—	Sri Lanka	Galpalama	*Brassica oleracea*	Gamini Herath, Anura Wijesekara
1	—	Sri Lanka	Sita Eliya	*Chrysanthemum* sp.	Gamini Herath, Anura Wijesekara
1	—	Sri Lanka	Sita Eliya	*Brassica juncea*	Gamini Herath, Anura Wijesekara
2	—	Israel	Gilat Exp. Station	*Solanum* sp.[b]	Phyllis Weintraub
1	—	Israel	Gilat Exp. Station	*Apium* sp.[b]	Phyllis Weintraub
1	—	Israel	Gilat Exp. Station	*Lactuca* sp.[b]	Phyllis Weintraub
1	—	Indonesia, W. Java	Garut	*Solanum* sp.	Merle Shepard
2	—	Indonesia, W. Java	Pangalengan	*Solanum* sp.	Merle Shepard

—, Indicates specimens for which mitCO data were presented in Scheffer (2000).
[a] In the case of lab colonies, the location given is the geographic area from which the original colony founders were taken.
[b] Flies swept or vacuumed from host plant; all others reared.

and Horowitz 1995). These reports are almost entirely based on observations that in certain areas, *L. huidobrensis* does not seem to attack crops that are favored in other areas (Spencer 1973), or that in some regions it is possible to control *L. huidobrensis* with insecticides while in other areas such control is difficult or impossible (Weintraub and Horowitz 1995). To date no controlled or parallel studies have been conducted on host preferences, host-associated performance, or insecticide resistance of flies from different geographic regions.

Recent phylogeographic investigation of variation in mitochondrial cytochrome oxidase sequences has shown that *L. huidobrensis* as currently defined contains two distinct monophyletic groups (Scheffer 2000). One group is composed of specimens from North America ("California Clade") and the other of specimens from South and Central America ("South America Clade") (Scheffer 2000). Although these geographically isolated clades show considerable divergence in mitochondrial sequence, no morphological differences are known to distinguish them. Scheffer (2000) suggested that these clades probably represent cryptic species, but without confirming evidence from either morphology or additional independent genes, no taxonomic changes were recommended.

The purpose of the current study was to investigate genetic variation and phylogenetic history of *L. huidobrensis* using DNA sequence data from two nuclear genes. We chose two gene regions, elongation factor-1α (EF-1α) and a beta-tubulin intron (β-tubulin). These two genes are predicted to evolve at different evolutionary rates and are presumed to be independent in location.

Materials and Methods

Specimens of *L. huidobrensis* were obtained from various hosts and locations around the world (Table 1). Flies were stored in 95–100% ethanol in an ultracold freezer before DNA extraction. DNA was extracted from individual fly specimens following the Blood and Body Fluid procedure of the QIAamp Blood Kit (QIAGEN, Valencia, CA). To obtain initial amplification of β-tubulin, we used the fruit fly versions of the primers Tub3–5′ (5′-GATCTGGAGCCCGGAACCATGGA) and Tub4–3′ (5′-ATGCGGTCGGGGTACTCCTCTCG) from Palumbi (1996). For all samples, a second amplification was performed using the first as template, the primer Tub4–3′, and one internal primer, Tub7–5′ (5′-ATCAGGAGCAGGCAATAACTGGG) exactly matching the *L. huidobrensis* sequence. This reamplification resulted in a single amplification product of length 256 bp. All polymerase chain reaction (PCR) amplifications were performed in an Eppendorf Mastercycler Gradient thermocycler (Eppendorf, Westbury, NY) with the following program: an initial denaturing step at 92 C for one minute; 35 cycles of 92 C for 30 s, 45 C for 30 s, 72 C for 1 min; and a final extension step of 72 C for 7 min. Sequencing reactions (see below) were performed with primers Tub4–3′ and Tub7–5′.

Table 2. Alignment of β-tubulin sequence data used in phylogenetic analysis

	N	Beta-tubulin 5′ exon sequence 5′ . . . 3′
CA1	20	AGGGTGCTGAATTAGTTGATAATGTTTTGGATGTTGTGCGTAAAGAATGTGAAAATTGTGACTGCCTGCAG
SA1	21	ARGGTGCTGAATTRGTTGATAATGTTTTGGATGTTGTGCGTAAAGAATGTGAAAATTGTGACTGCCTGCAG
Lbry	2	AAGGTGCTGAATTAGTTGATAATGTTTTGGATGTTGTGCGTAAAGAATGTGAAAATTGTGACTGCCTACAG
		Beta-tubulin intron sequence 5′ . . . 3′
		* * * * *
CA1	20	GTAAGCTTGTTTCACTTACAAGTAAATG-AAAATATTGAATTTTTTTTTTTAATAAG
SA1	21	GTAAACTTGTTTCACTTACAAGTAAATT-AAAATATTGA-TATTTTTTTTAAATA-G
Lbry	2	GTAAGCTTGTTTCACTTAAAAGGAATTTCAAGATATTGA-CATTATTTCTAAAAA-G
		Beta-tubulin 3′ exon sequence 5′ . . . 3′
		* *
CA1	20	GGCTTCCAATTGACTCACTCTTTGGGCGGCGGT
SA1	21	GGCTTTCAATTGACTCACTCTTTGGGCGGAGGT
Lbry	2	GGCTTCCAATTGACTCACTCTTTGGGAGGAGGT

Dashes indicate hypothesized gaps. (*) Indicates positions that differ between the California clade (CA1) and the South America clade (SA1). *Liriomyza bryoniae* sequence (Lbry) was used as the outgroup sequence.

To obtain amplification of elongation factor-1α, the primers EF-40 (5′-GTCGTGATCGGACACGTCGATTCCGG) and EF-53 (5′-GCGAACTTGCAAGCAATGTGAGC) were used to produce a single amplification product of ≈1088 bp. The PCR conditions were the same as those given above for beta-tubulin. Sequencing was performed with these original primers along with internal primers EF-46 (5′-TGAGGAAATCAAGAAGGAAG) and EF-50 (5′-ACTTCCTTCTTGATTTCCTC). Because EF-1α exhibited such a small amount of nucleotide variation, only one specimen, chosen haphazardly, per host-location sample was sequenced.

Sequencing reactions were performed using the BigDye Terminator Kit (Perkin-Elmer Applied Biosystems, Foster City, CA) according to the manufacturer's protocols with the modification that the volume of all reaction components was reduced by 75% so that the total volume of sequencing reactions was 5 μl. This alteration in protocol had no apparent effect on sequencing results. Sequencing samples were analyzed on an ABI 377 Automated DNA Sequencer (Perkin-Elmer Applied Biosystems). Sequences of both β-tubulin and EF-1α were confirmed by sequencing in both directions for all specimens for which data are presented with the exception of the β-tubulin intron data for the California clade. The β-tubulin intron for these specimens contained a poly-T region of at least 10 thymine residues through which we were unable to sequence (Table 2, 3′ area of intron). However, the unconfirmed sequences we obtained from either side of the poly-T region were identical across specimens, thus providing a good degree of corroboration.

Sequences were made into contigs and aligned by eye using Sequencher (Gene Codes, Ann Arbor, MI). Primer sequences were removed from the final alignments. Because of the small number of informative sites in each of the two datasets, they were combined into one analysis for this publication. However, separate analyses of each of the two datasets gives similar results and does not change the interpretation. Phylogenetic analysis of aligned datasets was performed using the heuristic search feature of PAUP* 4.0b2a (Swofford 2000) with random addition of taxa and 50 replicates. Gaps were treated as missing data in the analysis presented, although including them as a fifth character gives similar results. Bootstrap analysis of the final dataset was performed with 500 replicates. Because EF-1α data were available for only some taxa in the combined dataset, we present a tree for only those specimens for which we obtained sequences from both genes.

Results

Beta-tubulin sequence data were obtained from 41 *L. huidobrensis* specimens and two individuals of the outgroup *L. bryoniae.* Representative haplotypes have been deposited in GenBank under accession numbers AY035315-AY035318. The final alignment of 161 bp included a 57 bp intron. Within *L. huidobrensis,* haplotypes fell into two main groups (designated CA1 and SA1), and the alignment of these haplotypes along with that of the outgroup *L.bryoniae* is presented in Table 2. Alignment by eye was unambiguous except for the placement of a few gaps (Table 2). However, as can be seen in Table 2, the alignment we have chosen is conservative with regard to our question of interest in that it minimizes the number of differences seen between *L. huidobrensis* haplotypes.

Only one haplotype (CA1) was found in the 20 specimens sampled from California and Hawaii. The 21 specimens sampled from Central America, South America, and nonAmerican locations (i.e., from invasive populations) all shared the SA1 type of haplotype. The only variation observed within this haplotype group was two polymorphic sites within the exon. The SA1 haplotypes differed from the CA1 haplotype by 6 bp substitutions and two indels (Table 2). The uncorrected pairwise distance between the CA1 haplotypes and the SA1 haplotype is 9.2% for the intron region and 4.9% for the entire region (intron plus exon) (note: distance estimates do not include indels) (Table 3).

Table 3. Maximum uncorrected pairwise percent differences among DNA sequence data from three independent gene regions for the California clade (CA), the South America clade (SA), and *L. bryoniae* (Lbry)

	β-tubulin			EF-1α (894)	mitCO[a] (941)
	Intron[b] (57)	Exon (104)	Total (161)		
within CA clade	0	0	0	0	0.6
within SA clade	0	0.9	1.1	0.2	0.8
CA-SA	9.2	3.0	4.9	0.4	5.3
Lbyr-CA	18.5	2.7	9.7	2.1	8.6
Lbry-SA	15.8	3.9	7.8	2.1	8.1

The number of nucleotides obtained for each gene region is in parentheses.

[a] Mitochondrial data (mitCO) are from Scheffer (2000).

[b] Note that the percent differences in the Beta-tubulin intron do not take into account the indels hypothesized for this region.

These values are considerably larger than the variation observed within either group which ranged from 0–1.1% (Table 3).

Elongation factor-1α sequence data were obtained from 20 *L. huidobrensis* specimens and one specimen from the outgroup *L. bryoniae.* These sequences have been deposited in GenBank under accession numbers AY035319-AY035339. Pairwise distances between specimens were very low, ranging from 0% within the California clade to 2.1% between *L. huidobrensis* and *L. bryoniae* (Table 3). Across 894 bp of aligned coding region for EF-1α, five nucleotide sites varied within *L. huidobrensis.* Three of these sites involved nucleotide substitutions allowing the discrimination of the South American clade from the California clade. One site was variable within the South American clade and one site was variable within the California clade. All five variable sites involved transitions between the pyrimidines cytosine and thymine, and all occurred in third base pair codon positions.

Maximum parsimony analysis of the combined dataset of sequences from both gene regions resulted in 12 equally parsimonious trees, one arbitrarily chosen example of which is shown in Fig. 1. There were 13 parsimony informative sites, the consistency index was 0.93, and the consistency index excluding uninformative sites was 0.9. All of the equally parsimonious trees found that sequences from the California and Hawaii specimens formed one monophyletic group (Fig. 1, California Clade) while the South and Central American and nonAmerican specimens formed another monophyletic group (Fig. 1, South America Clade). These two groups correspond to the two monophyletic groups found using mitochondrial data (Scheffer 2000). In the current analysis of nuclear genes, bootstrap support was 100% for the California clade and 82% for the South America clade. The inclusion of gaps as a fifth character resulted in the same number of trees with slightly longer estimated branch lengths for the root and for the California clade, depending on the exact alignment used.

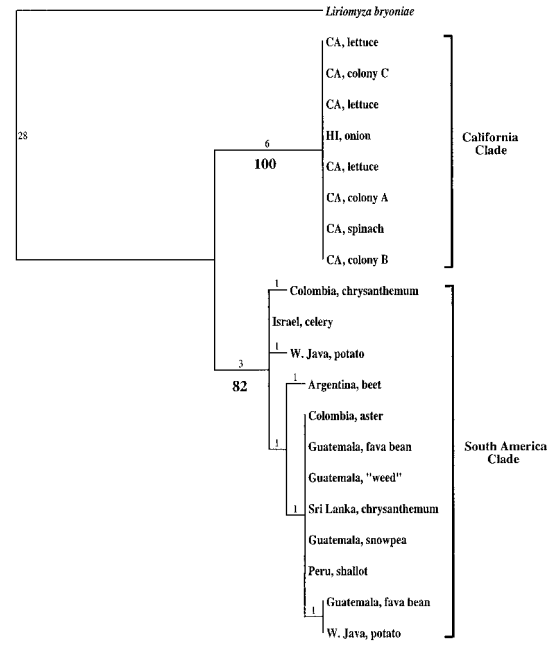

Fig. 1. One of 12 equally parsimonious phylograms from maximum parsimony analysis of combined EF-1α and beta-tubulin sequence data. Estimated branch lengths shown above branches, bootstrap values below.

Discussion

Results from the nuclear genes β-tubulin and EF-1α confirm the divergence previously observed within *L. huidobrensis* using mitochondrial cytochrome oxidase sequence data (Scheffer 2000). As with the mitochondrial data, the variation observed in β-tubulin between the two clades is considerably greater than variation seen within clades (Table 3). Within each clade, haplotypes from geographically disparate areas are nearly identical, indicating that gene flow or other homogenizing processes are operating. For example, samples from across the entire continent of South America only differ by a maximum of 1.1%. EF-1α is a highly conserved gene that is most commonly used in systematics to investigate much older divergences (Friedlander et al. 1992, 1994, Cho et al. 1995, Mitchell et al. 1997), although recently it was used to show uniformity between the tobacco aphid and the green peach aphid (Clements et al. 2000). As expected, this gene exhibited far fewer fixed differences between the *L. huidobrensis* clades than the more quickly evolving mitochondrial or intron regions studied. Nevertheless, the three fixed differences that were observed within EF-1α are enough to distinguish the two clades and to corroborate results from the other two gene regions.

In addition to confirming the findings reported in Scheffer (2000), the current study extends those results by increasing the sampling to include additional hosts and geographic locations within both North and South America. Specifically, specimens from three field populations of California flies were added (from

lettuce, spinach, and *Gypsophila*) as well as specimens from populations in Argentina (beets), Colombia (chrysanthemum and aster), and Peru (fava beans, lettuce, and shallots). In all cases, phylogenetic analysis of both β-tubulin and EF-1α sequences showed that these new samples belong to the clade predicted by the geographic location. In fact, the close correspondence between phylogeny and geography within areas of endemism argues strongly that divergence between the two clades is due to geographic isolation, with one group restricted to western North America and the other present only in South and Central America. There is currently no overlap between the distributions of the two clades within the endemic range. In fact, *L. huidobrensis* has never been reported from the southwestern United States or Mexico, and it was first reported from Central America only fairly recently (Costa Rica, Spencer 1983).

The evidence to date strongly suggests that the California and the South America clades, first identified by Scheffer (2000), each represent a distinct species. The sequence data from three independent gene regions (mitochondrial CO region, β-tubulin, EF-1α) are concordant and indicate a deep divergence between the clades. This divergence is in sharp contrast to levels of variation within clades which are highly homogeneous across their entire ranges, even across the South American continent. Levels of divergence observed in the mitochondrial CO region, the only gene of the three for which an insect molecular clock estimate is available, suggests that the California and the South America clades have been isolated for ≈2 million years (Scheffer 2000). Additionally, the level of divergence seen with the mitochondrial data is equal to or greater than that observed between some reproductively isolated agromyzid species (e.g., Scheffer and Wiegmann 2000).

We formally resurrect the name *L. langei* Frick (Frick 1951) for the California clade of *L. huidobrensis*-like flies currently found in California and Hawaii. The name *L. huidobrensis* (Blanchard) (Blanchard 1926) is now restricted to flies belonging to the South America clade. This includes *L. huidobrensis* in Central and South America and all of the introduced populations of *L. huidobrensis* sampled to date (Scheffer 2000, Scheffer et al. 2001).

Unfortunately, morphological characters useful in differentiating *L. langei* from *L. huidobrensis* are not currently known (Spencer 1973; S.J.S., unpublished data), although additional morphological studies are currently underway. These species can be readily distinguished once DNA sequence data are obtained from any of the three gene regions discussed here (for mitochondrial CO methods see Scheffer 2000). Additionally, a quick and fairly inexpensive PCR-restriction fragment-length polymorphism method has been developed to diagnose these two species without requiring sequence data (Scheffer et al. 2001). Because geography is an accurate predictor of species affiliation within areas of endemism, it is likely that flies that morphologically appear to be *L. huidobrensis* in California and Hawaii probably represent *L. langei* and those in South and Central America probably represent *L. huidobrensis.* However, until a morphological diagnosis is available, it is advisable that researchers confirm species identity using molecular methods. Currently, *L. langei* and *L. huidobrensis* are not known to be sympatric in any portion of their ranges, endemic or introduced, but given the rapid spread of *L. huidobrensis* within the past decade it is likely they will eventually be found in sympatry.

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References Cited

Avise, J. C. 2000. Phylogeography: the history and formation of species. Harvard University Press, Cambridge, MA.

Avise, J. C., and R. M. Ball. 1990. Principles of genealogical concordance in species concepts and biological taxonomy. Oxf. Surv. Evol. Biol. 7: 45–67.

Bartlett, P. W. 1993. Plant quarantine experience of *Liriomyza* spp. in England and Wales, pp. 23–30. *In* "*Liriomyza*" Conference on Leafmining Flies in Cultivated Plants, Montpellier, France.

Blanchard, E. 1926. A dipterous leafminer on *Cineraria*, new to science. Revta Soc. Entomol. Argent. 1: 10–11.

Blanchard, E. 1938. Descripciones y anotaciones de dipteros argentinos, Agromyzidae. An. Soc. Cient. Argent. 126: 352–359.

Blanchard, E. 1954. Sinopsos de los Agromyzidae argentinos (Diptera: Agromyzidae). Ministerio de Agricultura y Ganaderia, Buenos Aires.

Cheek, S., O. C. MacDonald, and P. W. Bartlett. 1993. Statutory action against *Liriomyza huidobrensis* (Blanchard) in the United Kingdom, pp. 79–86. *In* "*Liriomyza*" Conference on Leafmining Flies in Cultivated Plants, Montpellier, France.

Cho, A., A. Mitchell, J. C. Regier, C. Mitter, R. W. Poole, T. P. Friedlander, and S. Zhao. 1995. A highly conserved nuclear gene for low-level phylogenetics: Elongation Factor-1α recovers morphology-based tree for heliothine moths. Mol. Biol. Evol. 12: 650–656.

Clements, K. M., B. M. Wiegmann, C. E. Sorenson, C. F. Smith, P. A. Neese, and R. M. Roe. 2000. Genetic variation in the *Myzus persicae* complex (Homoptera: Aphididae): Evidence for a single species. Ann. Entomol. Soc. Am. 93: 31–46.

de Goffau, L.J.W. 1991. Liriomyza huidobrensis (Blanchard) (Diptera: Agromyzidae) a new economically important leaf miner in The Netherlands. Proc. Exp. Appl. Entomol. 2: 41–45.

Doyle, J. D. 1997. Trees within trees: Genes and species, molecules and morphology. Syst. Biol. 46: 537–553.

Frick, K. E. 1951. Liriomyza langei, a new species of leafminer of economic importance in California. Pan-Pac. Entomol. 21: 81.

Frick, K. E. 1958. Liriomyza dianthi n. sp., a new pest of carnations in California. Proceed. Entomol. Soc. Wash. 60: 1–5.

Frick, K. E. 1964. Liriomyza dianthi, a new synonym of *L. langei* Frick (Diptera: Agromyzidae). Pan-Pac. Entomol. 40: 11–12.

Friedlander, T. P., J. C. Regier, and C. Mitter. 1992. Nuclear gene sequences for higher level phylogenetic analysis: 14 promising candidates. Syst. Biol. 41: 483–490.

Friedlander, T. P., J. C. Regier, and C. Mitter. 1994. Phylogenetic information content of five nuclear gene sequences in animals: Initial assessment of character sets from concordance and divergence studies. Sys. Biol. 43: 511–525.

Frohlich, D. R., I. Torres-Jerez, I. D. Bedford, P. G. Markham, and J. K. Brown. 1999. A phylogeographical analysis of the *Bemisia tabaci* species complex based on mitochondrial DNA markers. Mol. Ecol. 8: 1683–1691.

Hall, H. G., and K. Muralidharan. 1989. Evidence from mitochondrial DNA that African honey bees spread as continual maternal lineages. Nature (Lond.) 339: 211–213.

Hoelzer, G. A. 1997. Inferring phylogenies from mtDNA variation: Mitochondrial-gene trees versus nuclear-gene trees revisited. Evol. 51: 622–626.

Mitchell, A., S. Cho, J. C. Regier, C. Mitter, R. Poole, and M. Matthews. 1997. Phylogenetic utility of elongation Factor-1a in Noctuoidea (Insecta: Lepidoptera): the limits of synonymous substitution. Mol. Biol. Evol. 14: 381–390.

Moore, W. S. 1995. Inferring phylogenies from mtDNA variation: mitochondrial-gene trees versus nuclear-gene trees. Evol. 49: 718–726.

Neigel, J. E., and J. C. Avise. 1986. Phylogenetic relationships of mitochondrial DNA under various demographic models of speciation., pp. 515–534. *In* E. Nevo and S. Karlin [eds.], Evolutionary process and theory. Academic, New York

Palumbi, S. R. 1996. Nucleic acids II: the polymerase chain reaction., pp. 205–247. *In* D. M. Hillis, C. Moritz, and B. K. Mable [eds.], Molecular systematics. Sinauer, Sunderland, MA.

Scheffer, S. J. 2000. Molecular evidence of cryptic species within *Liriomyza huidobrensis* (Diptera: Agromyzidae). J. Econ. Entomol. 93: 1146–1151.

Scheffer, S. J., and B. M. Wiegmann. 2000. Molecular phylogenetics of the holly leafminers (Diptera: Agromyzidae: *Phytomyza*): Species limits, speciation, and dietary specialization. Mol. Phylogenet. Evol. 17: 244–255.

Scheffer, S. J., A. Wijesekara, D. Visser, and R. H. Hallett. 2001. A molecular method to distinguish *Liriomyza huidobrensis* from *L. langei* (Diptera: Agromyzidae) applied to three recent leafminer invasions. J. Econ. Entomol. (in press).

Shepard, B. M., A. Braun, and Samsudin. 1996. Liriomyza huidobrensis: a newly introduced pest of vegetables. Palawija Veg. IPM Newsl. 1: 2.

Slade, R. W., and C. Moritz. 1998. Phylogeography of *Bufo marinus* from its natural and introduced ranges. Proc. R. Soc. Lond. B. 265: 769–777.

Spencer, K. A. 1973. Agromyzidae (Diptera) of economic importance. W. Junk, The Hague.

Spencer, K. A. 1983. Leaf mining Agromyzidae (Diptera) in Costa Rica. Rev. Biol. Trop. 31: 41–67.

Spencer, K. A. 1990a. Host specialization in the world Agromyzidae (Diptera). Kluwer, Dordrecht.

Spencer, K. A. 1990b. Keeping track of *L. huidobrensis.* Grower. 113: 15.

Swofford, D. L. 2000. PAUP*. Phylogenetic analysis using parsimony (*and other methods). Sinauer, Sunderland, MA.

Villablanca, F. X., G. K. Roderick, and S. R. Palumbi. 1998. Invasion genetics of the Mediterranean fruit fly: variation in multiple nuclear introns. Mol. Ecol. 7: 547–560.

Weintraub, P. G., and A. R. Horowitz. 1995. The newest leafminer pest in Israel, *Liriomyza huidobrensis.* Phytoparasitica 23: 177–184.

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